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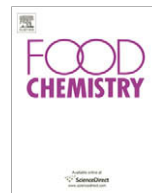

Superoxide and hydroxyl radical scavenging actions of botanical extracts of *Wagatea spicata*

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ABSTRACT

Wagatea spicata, candy corn plant, a woody legume shrub, widespread medicinal plant found in Western Ghat of India has significant abilities to scavenge highly reactive free radicals. Shade dried leaf, bark and flower powder of this plant has been extracted with water and fractionated with different solvents. Extracts and their solvent fractions were found to be good scavengers of superoxide and hydroxyl radicals. Free radical scavenging action of *W. spicata* is due to its rich phenolic and flavonoid contents. Bark and leaf extracts showed significant scavenging action against superoxide radicals, where as flower extracts efficiently inhibited hydroxyl radicals.

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1. Introduction

Reactive Oxygen Species (ROS) have been implicated in more than 100 diseases from Malaria to Haemorrhagic shock to AIDS (Alho & Leinonen, 1999). Atmospheric pollutants and radiations have become the main sources of free radicals. Oxidative stress causes various forms of tissues damage and inflammation, and plays an important role in the development of several degenerative changes in cells and tissues, which ultimately lead to several degenerative disorders. Bodily defenses are not completely efficient in preventing on going oxidative damage to DNA, lipids and proteins. Dietary antioxidants, vitamins, flavonoids, plant phenolics, herbal formulations and Ayurvedic preparations are very essential in protecting against oxidative stress (Weiss & Landauer, 2000). Antioxidant supplementation constitutes important defence against variety of diseases and environmental stresses.

Wagatea spicata Wt. Syn. *Moullva spicata* (Dalz) (Family: Fabaceae) is commonly known as the candy corn plant, a small prickly, woody ornamental plant with long flaming spikes. This legume shrub is one of the most widespread medicinal plants in the Western Ghat forests of India. The whole plant possesses medicinal properties, and is useful in the treatment of various ailments such as fever, cough, gastrointestinal disorders, skin ailments etc., as reported by local people as they are using regularly roots, leaves and bark of the plant. Bark is used as an application for skin diseases. Root extract of *W. spicata* has a high phagocytic co-efficient and was found to be effective against skin infections (Behl & Tripathi, 1975).

In the present study, the free radical scavenging efficacy of plant material was evaluated with respect to superoxide and hydroxyl radicals. The aqueous extract of leaf, bark and flower of *W. spicata* were subjected to different solvent fractionation. Crude aqueous extracts were analysed for phenolic and flavonoid contents.

2. Materials and methods

2.1. Plant material

W. spicata plant materials, leaves, bark and flowers were collected soon after monsoon season from Western Ghats region of India.

2.2. Chemicals

Thiobarbituric acid (TBA) from Sigma Chemical Company (St. Louis, MO). Phenazine methosulphate, deoxyribose, reduced nicotinamide adenine dinucleotide (NADH), gallic acid, quercitin and other chemicals were of analytical grade purchased from SRL Research chemicals, India.

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2.3. Preparation of aqueous extract

Leaves, bark and flowers of *W. spicata* were collected, dried in shade and powdered. The powder was used for extraction. 100 g of plant material powder was refluxed with 750 ml of double distilled water (DDW) for 1 h at 75–80 °C, cooled and filtered. This was repeated in three trials; extracts were pooled and evaporated using a Lyophiliser (Geetha, Kedlaya, & Vasudevan, 2003). The extract yield was 16% in leaf, 30% in bark and 35% in flower.

2.4. Fractionation of extract

Leaf, bark and flower aqueous extracts of *W. spicata* were re-extracted with organic solvents ranging from polar to non-polar in succession. They were successively re-extracted with petroleum ether (60–80 °C), diethyl ether, ethyl acetate, methanol and water (Geetha et al., 2003; Suffness & Douros, 1979). The final yield of solvent free fractions of the aqueous extract of leaf, bark and flower was measured. No fractions yielded in petrol ether and diethyl ether solvent. Solvent fractions of ethyl acetate, methanol and water solvents were present with respect to leaf and bark, only methanol and water fractions in flower extract. Relative proportions of fractions are given in Table 1.

2.5. Superoxide scavenging activity

Superoxide scavenging activities of the compounds were determined by monitoring the competition of those with NBT for the superoxide anion generated by the PMS–NADH system (Liu, Ooi, & Chang, 1997). Superoxide radicals were generated in 1 ml 20 mM Tris–HCl buffer pH 8.0 containing 0.05 mM nitroblue tetrazolium (NBT), 0.01 mM phenazine methosulphate (PMS) and test compounds were preincubated for 2 min. The reaction was initiated by the addition of 0.078 mM NADH. Blue chromogen, formed due to NBT reduction, was read at 560 nm. Results were expressed as percentage of inhibition of superoxide radicals.

2.6. Hydroxyl radical scavenging activity

Hydroxyl radicals were measured by the deoxyribose method (Halliwell, Gutteridge, & Aruoma, 1987). Hydroxyl radicals generated by ferric-ascorbate–EDTA–H_2O_2, which attacks on deoxyribose to form products called thiobarbituric acid reactive substances (TBARS), which upon heating with TBA at low pH yield pink chromogen. The hydroxyl scavenger, when added, competes with deoxyribose for hydroxyl radicals and decreases TBARS formation and pink chromogen (Elizabeth & Rao, 1990). The reaction mixture containing 3 mM deoxyribose, 0.1 mM ferric chloride, 0.1 mM EDTA, 0.1 mM ascorbic acid and 2 mM H_2O_2 in 20 mM phosphate buffer pH 7.4 was added to various concentrations of test compounds. After incubating for 30 min at 37 °C, the reaction mixture was added to 0.5 ml of 5% trichloroacetic acid and 0.5 ml of 1% TBA to yield a final volume 3 ml. The reaction mixture was kept in a boiling water bath for 30 min, cooled and the absorbance was measured at 532 nm. Scavenging action was expressed as inhibitory concentration of test compound required to produce 50% inhibition of hydroxyl radicals i.e. IC_{50} values.

2.7. Estimation of the total phenolic contents in the Wagatea extracts

Phenol in alkaline medium reacts with phosphomolybdic acid of Folin–Ciocalteau's reagent producing blue colour complex. The amount of total phenolics in the extracts was determined by Folin–Ciocalteau's reagent (McDonald, Prenzler, Autolovich, & Robards, 2001). A dilute plant material extract 0.5 ml of 1:0.5 mg ml^{-1}) or gallic acid (standard phenolic compound) was mixed with Folin–Ciocalteau's reagent (5 ml, 1:10 diluted with distilled water) followed by addition of aqueous Na_2CO_3 (4 ml, 1 M). After incubating the reaction mixture at RT for 15 min and total phenols were determined colourimetrically at 765 nm. The standard curve of gallic acid monohydrate was prepared using 0, 50, 100, 150, 200, 250 mg L^{-1}. The total phenolic content of the extracts was expressed in terms of mg gallic acid equivalent (GAE)/ g dry weight of the plant extract.

2.8. Estimation of the total flavonoid contents in the Wagatea extracts

An aluminium chloride colourimetric method was used for flavonoids determination (Chang, Yang, Wen, & Chern, 2002). Each extract (0.5 ml of 1:0.5 mg ml^{-1}) in methanol were separately mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminium chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. It remained at room temperature for 30 min; the absorbance of the reaction mixture was measured at 415 nm with a double beam Genesis UV Spectrophotometer. The calibration curve was prepared by preparing quercetin solutions at concentrations 12.5 to 100 g ml^{-1} in methanol.

3. Results and discussion

3.1. Bark and leaf extracts of **W.** spicata are potent scavenger of superoxide radicals

W. spicata leaf, bark and flower *extracts* showed a dose dependent inhibition of superoxide radicals (Fig. 1). Crude bark extract was found to be a better scavenger (IC_{50} at 15 μg) than leaf and flower extracts (Table 2). Further methanol and water fraction of bark extract contained the active inhibitor of superoxide (IC_{50} at 13 μg and 14 μg, respectively). The crude extract of leaf showed better scavenging action than its fractions, where as water and methanol fractions of flower showed better scavenging action (IC_{50} at 30 μg and 20 μg, respectively) than the crude extract. IC_{50} values of all these compounds were greater than that of ascorbic acid where IC_{50} was achieved at 5 μg concentration (Geetha et al., 2003).

3.2. Flower and bark extracts of W. spicata are potent scavenger of hydroxyl radicals

The ability of *W. spicata* leaf, bark and flower extracts and their fractions to scavenge hydroxyl radicals was measured by studying competition between deoxyribose and test compounds for hydroxyl radical generated from ferric-ascorbate–EDTA–H_2O_2 system. Hydroxyl radicals attack deoxyribose starting a set of reactions,

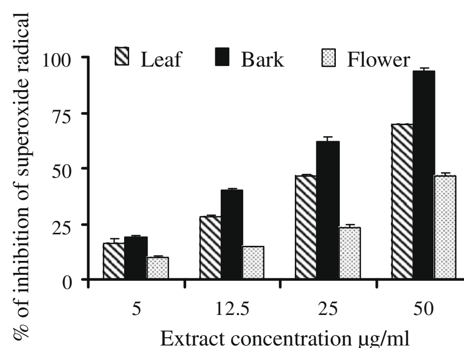

Fig. 1. Super oxide radical scavenging action of aqueous extract of leaf, bark and flower material of *W. spicata*. (Values are mean ± SEM of three separate experiments.)

which eventually results in TBARS formation. When a molecule scavenges hydroxyl radicals the TBARS formation is decreased. There is a concentration dependant inhibition of hydroxyl radical by *W. spicata* extracts and their fractions (Fig. 2). The crude flower extract was found to be a better scavenger than bark and leaf extracts (IC_{50} at 1.5 µg concentration) (Table 3). Among the fractions, ethyl acetate and methanol fractions of leaf and bark extracts inhibited TBARS formation at very low concentration with IC_{50} values at 0.05 µg, 0.5 µg, 0.5 µg and 1.2 µg, respectively.

3.3. Flower extract of Wagatea contains very high amount of total phenolics

The amount of total phenolics of the extracts was expressed in terms of gallic acid equivalents. The amount of total phenolics varied widely in *Wagatea* plant materials. Flower extracts had relatively high values of phenolic content (226.73 mg gallic acid equivalent/g of dry weight of crude extract) (Table 4). Bark contained 127.3 mg gallic acid equivalent/g of dry weight of crude extract.

3.4. Leaf extract of Wagatea contains more flavonoid content

The total amount of flavonoids in the extracts was expressed in terms of quercetin equivalents. The amount of total flavonoids varied widely in *Wagatea* plant materials (Table. 4). The leaf extract had relatively high amounts of flavonoids (12.7 mg quercetin equivalent/g of dry weight of crude extract. Bark contained 8.8 mg quercetin equivalent/g of dry weight of crude extract), but the flower extract contained the lowest value of flavonoid content (4.5 mg quercetin equivalent/g of dry weight of crude extract).

The present study reveals that *W. spicata* extracts and their fractions are potent scavenger of deleterious free radicals, such as $O^{\cdot-}$ and $OH^{\cdot-}$, at very low concentrations. Superoxide is the first reduction product of molecular oxygen, a highly toxic radical, the most abundantly produced in all aerobic cells by several enzymatic and non-enzymatic pathways, attacks a number of biological molecules and leads to unfavourable alterations of biomolecules including DNA (Waris & Alam, 2004). It also forms an important source of other deleterious radicals such as hydroxyl and hydroperoxides, which initiate free radical chain reactions (Halliwell & Gutteridge, 1990).

Free radical scavenging action of *W. spicata* can be attributed to its rich phenolic and flavonoid contents. The phenolic rich flower extract brought significant inhibition of hydroxyl radicals whereas phenolic and flavonoid rich bark was found to be good scavenger of superoxide radicals. Ethyl acetate and methanol fractions of bark and leaf extract efficiently scavenged hydroxyl radicals at very low concentrations.

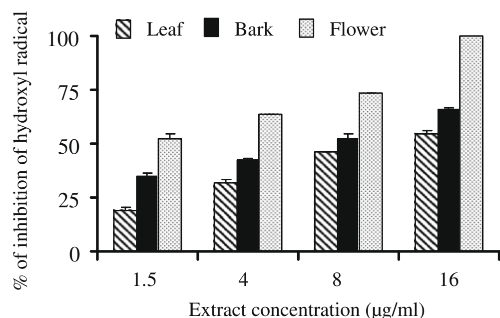

Fig. 2. Hydroxyl radical scavenging action of aqueous extract of leaf, bark and flower material of *W. spicata*. (Values are mean ± SEM of three separate experiments.)

Table 1
Relative proportion of solvent fractions yielded during fractionation of crude leaf, bark and flower extracts of *W. spicata* expressed as percentage of total amount of crude extract used for the fractionation (average of two trials).

Solvent fractions	Leaf	Bark	Flower
Petrol ether	0	0	0
Diethyl ether	0	0	0
Ethyl acetate	0.65	0.75	0
Methanol	60.1	64.45	33.7
Water	26.1	25.9	38.9
Undissolved	13.15	8.9	27.4

Table 2
Super oxide radical scavenging action of *W. spicata* aqueous extract of leaf, bark and flower and their fractions expressed as IC_{50} values (concentration in micrograms (µg/ml) needed for 50% inhibition).

Extract	Leaf	Bark	Flower
Crude extract	28 ± 0.64	15 ± 2.4	55 ± 1.23
Ethyl acetate fraction	125 ± 0.4	40 ± 1.3	–
Methanol fraction	75 ± 0.5	13 ± 0.4	20 ± 0.3
Water fraction	50 ± 2.4	14 ± 3	30 ± 0.3

Table 3
Hydroxyl radical scavenging action of *W. spicata* aqueous extract of leaf, bark and flower and their fractions expressed as IC_{50} values (concentration in micrograms (µg/ml) needed for 50% inhibition).

Extract	Leaf	Bark	Flower
Crude extract	8.7 ± 0.06	4.6 ± 0.6	1.5 ± 0.8
Ethyl acetate fraction	0.05 ± 0.4	0.5 ± 0.1	–
Methanol fraction	0.5 ± 0.4	1.2 ± 0.5	20 ± 0.007
Water fraction	8.33 ± 0.7	16.67 ± 0.03	16.67 ± 0.03

Table 4
Total phenolic and flavonoid content of leaf, bark and flower extracts of *W. spicata* (values are mean ± SEM of three separate experiments).

Plant material	Total phenolics	Total flavonoids
Leaf	47 ± 3	13 ± 1
Bark	127 ± 1	9 ± 1
Flower	227 ± 2	4 ± 0

The antioxidant activity of phenolics is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donators, and singlet oxygen quenchers. In addition, they have a metal chelating potential (Rice-Evans, Miller, Bolwell, Bramley, & Pridham, 1995). The presence of conjugated ring structures and hydroxyl groups allows phenolics to actively scavenge free radicals; carboxylic acid groups inhibit lipid peroxidation and are also known for their ability to inhibit prooxidant enzymes. (Decker, 1995).

Naturally occurring Polyphenols and flavonoids have been shown to prevent lipid peroxidation, LDL oxidation, development of Atherosclerosis and heart disease. (Fuhrman, Rosenblat, Hayek, Coleman, & Aviram, 2000; Geetha, Kedlaya, & Vasudevan, 2004; Hertog, Feskens, Holiman, Katan, & Kromhout, 1993; Samak, Rao, Kedlaya, & Vasudevan, 2007). In several studies it was concluded that plant flavonoids which show antioxidant activity *in vitro* also function as antioxidants *in vivo* (Geetha et al., 2004; Shimoi, Masuda, Shen, Furugori, & Kinae, 1996). From the present study it is evident that *W. spicata* extracts and fractions are good scavengers of reactive free radicals. Many antioxidants similar to ascorbate and phenolic compounds, possess prooxidant properties (Narla & Rao, 1995). But *W. spicata* extracts and their fractions are free from such prooxidant properties.

Crude extracts of fruits, herbs, vegetables, cereals, and other plant materials rich in phenolics are increasingly of interest in the food industry because they retard oxidative degradation of lipids and thereby improve the quality and nutritional value of food (Marja et al., 1999). In recent years there is a growing interest in antioxidant supplements for the prevention of many diseases. In this context *W. spicata* can be exploited for its impressive free radical scavenging activities. Ingestion of alcohol-free red wine or a phenolic compound mixture extracted from red wine has been shown to improve the antioxidant status of plasma in humans (Carbonneau, Léger, Descomps, Michel, & Monnier, 1998; Serafini, Maiani, & Ferro-Luzzi, 1998). Hence phenolic rich *Wagatea* can be explored in different directions to tackle several oxidative stress induced ailments.

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